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Stably expressed D-genome-derived HMW glutenin subunit genes transformed into different durum wheat genotypes change dough mixing properties

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Abstract Durum wheat (*Triticum turgidum L.* var. *durum*) is traditionally used for the production of numerous types of pasta, and significant amounts are also used for bread-making, particularly in southern Italy. The research reported here centres on the glutenin subunits 1Dx5 and 1Dy10 encoded by chromosome 1D, and whose presence in hexaploid wheats is positively correlated with higher dough strength. In order to study the effects of stable expression of the 1Dx5 and 1Dy10 glutenin subunits in different durum wheat genotypes, four cultivars commonly grown in the Mediterranean area ('Svevo', 'Creso', 'Varano' and 'Latino') were co-transformed, via particle bombardment of cultured immature embryos, with the two wheat genes *Glu-D1-1d* and *Glu-D1-2b* encoding the glutenin subunits, and a third plasmid containing the *bar* gene as a selectable marker. Protein gel analyses of T_1 generation seed extracts showed expression of one or both glutenin genes in four different transformed durum wheat plants. One of these transgenic lines, DC2-65, showed co-suppression of all HMW-GS, including the endogenous ones. Transgene stability in the transgenic lines has been studied over four generations (T_1–T_4). Fluorescence in situ hybridization (FISH) analysis of metaphase chromosomes from T_4 plants showed that the integration of transgenes occurred in both telomeric and centromeric regions. The three plasmids were found inserted at a single locus in two lines and in two loci on the same chromosome arm in one line. The fourth line had two transgenic loci on different chromosomes: one with both glutenin plasmids and a different one containing only the construct with the gene encoding the 1Dy10 glutenin subunit. Segregation of these two loci in subsequent generations allowed establishment of two sublines, one containing both 1Dx5 and 1Dy10 and the other containing only 1Dy10. Small-scale quality tests showed that accumulation of Dx5, Dy10 or both in transgenic durum wheat seeds resulted in doughs with stronger mixing characteristics.

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Introduction

Wheat is an important crop and, among the food crops, is one of the most abundant sources of energy and protein for the world's population. Ninety-five percent of wheat grown today is the hexaploid type (*Triticum aestivum L.*), mainly used for the preparation of bread. The remaining 5% is durum wheat (*Triticum turgidum* var. *durum*). Durum wheat is a tetraploid species (AABB) adapted to several environments worldwide and principally used for making pasta and biscuits (Patnaik and Khurana 2001), although currently a significant amount is also used for bread-making in southern Italy. Although cereal proteins do not constitute the major fraction of the kernel by weight, they play a very important role in the final end-use of the grain.

Amongst the cereals, the flour of hexaploid wheat has the best capability of forming leavened bread. This superiority stems from the structure and composition of its seed storage proteins, which upon hydration can interact to form gluten, an insoluble, but highly hydrated, visco-elastic aggregate that endows the wheat dough with its unique properties. Although about half of wheat seed storage proteins participate in gluten network formation, biochemical and genetic evidence has demonstrated that high molecular weight glutenin subunits (HMW-GS) play a major role in determining the viscoelastic properties that underlie bread dough formation (reviewed and referenced in Shewry et al. 2003). The HMW-GS are necessary to create strong doughs that can trap tiny bubbles of carbon dioxide gas formed by yeast during proofing, thereby enabling the dough to rise and form high quality leavened breads. Increasing understanding of the molecular basis of dough visco-elasticity would thus help in developing strategies for minimizing the effect of unfavourable environmental factors on wheat end-use quality.

Genetic transformation has played a key role in gaining and applying knowledge of the roles of HMW-GS in wheat end-use properties (Vasil and Anderson 1997; reviewed and referenced in Jones 2005; Wieser et al. 2005; Blechl et al. 2007), but the production of transgenic lines is not sufficient to introduce biotechnology into production agriculture. Reliable and stable expression of transgenes as well as the characterization and field adaptation of transgenic lines are prerequisites for the successful application of gene technology. Many papers have reported significant variability in the behaviour of the same transgene in different lines (e.g., Iglesias et al. 1997). Loci that appear to be stably expressed initially can become progressively silenced over several generations (Srivastava et al. 1996). The stability and the behaviour of transgenes are influenced by several factors, such as chromosomal location, transgene copy number and arrangement, and interaction with the host genotype. The aim of the present work was the production of stable transgenic durum wheat lines expressing both 1Dx5 and 1Dy10 glutenin subunits. At the same time, we wanted to investigate HMW-GS transgene expression and inheritance in different durum wheat genotypes. We also wanted to assess the effects of introduction of the D-genome-derived subunits on field adaptation and end-use properties of durum wheats. We therefore carried out transformation experiments by the biolistic method of four durum wheat cultivars 'Svevo', 'Creso', 'Varano', and 'Latino' with the two bread wheat D-genome genes encoding the 1Dx5 and 1Dy10 glutenin subunits, and a third plasmid containing the *bar* gene as a selectable marker. A detailed analysis of the expression and stability of the transgenes was conducted on a set of four transgenic lines from the T_1 to T_4 generations. In addition, we have applied FISH analyses on T_4 homozygous lines in order to determine the number of inserted transgene loci and their chromosome position. The transgenic wheat lines were grown in the field to obtain sufficient seed for small-scale quality analyses.

Materials and methods

Embryo isolation and culture initiation medium

Immature seeds 15–18 days post-anthesis from durum wheat *cvs*. 'Svevo', 'Creso', 'Latino' and 'Varano' were surface-sterilized with 70% ethanol for 5 min and 1.05% sodium hypochlorite (20% bleach) for 15 min, then rinsed in sterile water.

Immature embryos that varied in size between 0.8 and 1.5 mm in diameter were aseptically excised under a stereo dissecting microscope and placed with the scutellar portion of the embryo exposed on a solid MS medium. The basal callus induction and maintenance medium, MS, contained the inorganic components of Murashige and Skoog (1962), 150 mg/l L-asparagine, 0.50 mg/l Thiamine-HCl, 40 g/l maltose, 3.5 g/l Phytagel, and 1 mg/l 2,4-D (Sigma, St. Louis, MO).

Transformation procedure and plasmid DNA

Only embryogenic calli were used for transformation. Calli were co-transformed with two plasmids (BluescriptKS+\−) containing the wheat genes that encode the 1Dx5 and 1Dy10 glutenin subunits. Details on the D genes are reported by Anderson et al. (1989). A third plasmid *Ubi:bar* containing the selectable *bar* gene, which confers resistance to the herbicide bialaphos under the control of the maize *Ubiquitin1* promoter, was used as a selectable marker. Bombardments were carried out using a PDS 1000\He particle gun (BioRad, Richmond, CA). Equimolar amounts of each plasmid DNA, equivalent to 12.5 µg of *Ubi:bar*, were precipitated onto 1 µm diameter gold particles immediately prior to bombardment as described by Weeks et al. (1993) and accelerated at 1100 psi pressure in a vacuum of 26 in. (66 cm) Hg into calli formed from embryos cultured for one week. After gene delivery, calli were incubated in the dark at 25°C for 3 weeks with weekly transfers to MS basal callus induction medium with 2.5 g/l of phytagel.

Selection of transgenic plants

Bialaphos selection was carried out by moving calli to selection/regeneration medium consisting of MS basic medium containing 0.2 mg/l 2,4-D and supplemented with 3 mg/l of bialaphos (Meiji Seika Kasha, Tokyo, Japan). Following growth at 25°C with 16 h of light and 8 h of darkness daily for 3 weeks, the regenerated shoots were transferred for rooting induction to Pyrex culture test tubes (25 × 150 mm) containing 18 ml of half-strength MS medium lacking hormones and supplemented with 3 mg/l of bialaphos.

Plantlets that showed healthy growth under selection were transferred from rooting media to pots containing Sunshine #1 soil mixture (Weeks et al. 1993), incubated in a growth chamber for about 2 weeks with decreasing humidity, and transferred to the greenhouse, with supplementary lighting provided for 16 h by 1000 W sodium vapour lamps and with day temperatures of 24°C and night temperatures of 17°C.

Molecular analysis

Genomic DNA of durum wheat shoots from regenerated plants classified as transformed and from non-transgenic control shoots of *cvs*. 'Svevo', 'Creso', and 'Varano' was isolated according to Dellaporta et al. (1983). In order to confirm the presence of *1Dx5* and *bar* genes in the putative transformants, fragments were amplified from genomic DNA with the following *1Dx5* and *bar* (He et al. 1999) primer pairs:

Dx5-1 = CGTCCCTATAAAAGCCTAGC
Dx5-2 = AGTATGAAACCTGCTGCGGAC
BAR-1 = GTCTGCACCATCGTCAACC
BAR-2 = GAAGTCCAGCTGCCAGAAAC

No primers are available for the PCR analysis of *Dy10* gene. PCR analyses were carried out in 25 µl reaction mixtures, each containing 100 ng template DNA, 2 µm of each primer pair, 200 µm of each dNTP, 2.5 mm $MgCl_2$, 10 mm Tris–HCl, pH 8.3, 10 mm KCl, and 1 unit of *Taq* DNA polymerase. Amplifications were conducted in a Perkin Elmer DNA Thermal Cycler with the following protocol: initial denaturation was at 95°C for 3 min, followed by 35 cycles of [95°C for 1 min, 60°C for 1 min, 72°C for 2 min] and then a final extension at 72°C for 10 min. The amplification products were resolved on 1.5% agarose gels and stained with ethidium bromide.

SDS-PAGE analysis of seed proteins

Protein extracts from endosperms were analysed by SDS-PAGE for changes in HMW-GS content. Total proteins were extracted from single half grains with 25 µl/mg of 63 mM Tris–HCl buffer, pH 6.8, containing 2% (v/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.002% (v/v) bromophenol blue and separated by SDS-PAGE, using the conditions described in Blechl et al. (2007).

Approximately equal amounts of protein, as estimated by visual examination of preliminary gels of the same extracts, were loaded in each lane.

Inheritance and expression of transgenes from T_1 to T_4

T_1 seeds were obtained by self-pollination of primary transformed durum wheat cvs. 'Svevo', 'Creso' and 'Varano' T_0 plants. The analyses of inheritance and expression of *1Dx5*, *1Dy10* and *bar* genes were conducted in each transgenic line from T_1 to T_4 generations. For each T_1 transgenic line, segregation analysis was conducted on 7–14 seeds. Plants showing the expression of *1Dx5* and/or *1Dy10* were grown in the greenhouse and harvested in bulk to produce T_2 seeds; subsequently T_3 and T_4 progenies were analysed in order to find homozygous lines and monitor the stability of the transgenes. The distal endosperm from progeny seeds containing storage proteins were analysed by SDS-PAGE for expression of the HMW-GS transgenes *1Dx5* and *1Dy10*, while the corresponding proximal parts containing the embryo were germinated on MS medium supplemented with 3 mg/l of bialaphos to detect the expression of *bar* gene.

FISH

Transgene loci of four independent transgenic lines were characterized by fluorescence in situ hybridization (FISH). Root-tips of transgenic T_4 homozygous plants were removed and placed in ice-water for 24 h and then fixed in 3:1 (v:v) ethanol:glacial acetic acid. Meristematic portions of root tips were dissected onto microscope slides and squashed in the presence of 45% acetic acid before coverslips were removed after freezing on dry ice. The preparations were then dried, pretreated with Pepsin-HCl to eliminate cytoplasmic proteins, and dehydrated in 70–90–100% ethanol before being used for FISH. FISH on metaphase chromosomes was done according to previously published protocols (Pedersen et al. 1997). In situ hybridization was performed using as probes whole plasmids *pKS-1Dy10*, *pKS+1Dx5* and *Ubi:Bar* directly labelled respectively with Cy5 (Blue), Cy3 (Red) and FITC (Green) (Perkin-Elmer Wellesley, MA, USA) by nick-translation according to the manufacturer's protocols. Digital images were obtained using a Leica DMRXA epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments, Acton UK). Cy5 (Blue), Cy3 (Red) and FITC (Green) fluorescence signals, detected with specific filters, were recorded separately as gray-scale images. Pseudocoloring and merging of images were performed using Adobe Photoshop (San Jose, California) software.

Field trials

Homozygous seeds from lines DC2-10 (T_3), DC2-65 (T_3), DV1-4 (T_2) were increased in a field growth in the summer of 2004. In the summer of 2005, a small field trial was conducted in North Dakota as a randomized block design with three replicates for each of these lines, as well as for their untransformed parents, 'Creso', 'Varano' and 'Svevo', and North Dakota varieties 'Alzada', 'Lebsock' and 'Rugby'. Also included in the trial were homozygous T_5 and T_6 seeds from greenhouse grown plants of the subline of DS2-127 that contained only Dy10.

Quality analyses

One hundred grams of wheat seed harvested from individual plots were milled and analyzed for protein contents by nitrogen combustion, and by SDS sedimentation tests and the 2-gram mixograph, using the procedures described in Blechl et al. (2007). The SDS sedimentation tests and protein determinations were done in triplicate for each plot sample and the 2-gram mixographs were done in duplicate.

References to a company and/or product by the USDA are only for purposes of information and do not imply approval or recommendation of the product to the exclusion of others that may also be suitable.

Results

Production of transgenic plants

Four durum wheat varieties ('Svevo', 'Creso', 'Latino', 'Varano') were used for transformation experiments. A total of 2105 scutellar-derived embryogenic calli from 'Svevo', 1350 from 'Creso', 1120 from 'Latino' and 100 from 'Varano' were co-bombarded with equimolar amounts of three separate plasmids containing genes encoding 1Dx5 and 1Dy10

Table 1 Number of transgenic plants and transformation efficiency obtained in three transformation experiments (I–III) of four durum wheat cultivars with HMW-glutenin genes *1Dx5 and 1Dy10* and the *bar* gene as selectable marker gene

Cultivar	Bombardment (I–III)	Calli (N°)	Transgenic plants (N°)	Transformation efficiency (%)
‘Svevo’	I	600	0	0
	II	737	1	0.1
	III	550	0	0
‘Latino’	I	750	0	0
	II	600	0	0
‘Creso’	I	350	0	0
	II	652	2	0.3
‘Varano’	I	100	1	1

and the *bar* gene conferring bialaphos-resistance in eight different bombardment experiments (Table 1). Selection pressure was applied by adding 3 mg/l of bialaphos to the shoot and root regeneration media. PCR primers were used to confirm the presence of the *bar* transgene(s). We also attempted to detect the 1Dx5 transgene by PCR, but due to the high level of sequence homology among HMW-GS genes, the primers designed for the 1Dx5 subunit gene could not distinguish it from the native HMW-GS genes, at least one of which gave a band of similar size. Therefore, we identified transgenic lines by changes in their seed storage proteins revealed by SDS-PAGE of T_1 endosperm extracts. Four independently derived plants showed changes in their seed HMW-GS composition (Fig. 1). Two transgenic lines (named DC2-10 and DC2-65) were derived from cultivar

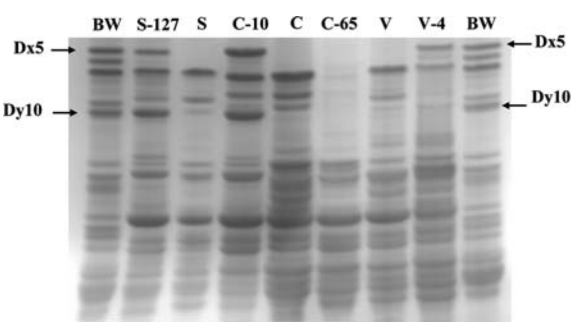

Fig. 1 SDS-PAGE of endosperm proteins from seeds of transformed lines DS2-127 (S-127), DC2-10-4 (DC-10), DC2-65 (C-65) and DV1-4C (V-4), non-transformed bread wheat cultivar Bobwhite (BW), and non-transformed durum wheat cultivars ‘Svevo’ (S), ‘Creso’ (C) and ‘Varano’ (V). Lines DC2-10 and DS2-127 express the transgene encoded subunits Dx5 and Dy10. Line DV1-4C expresses transgene-encoded subunit Dx5 only. Line DC2-65 shows transgene mediated co-suppression of all HMW-GS including endogenous genes

‘Creso’, and one each (named DV1-4 and DS2-127 respectively) was derived from cultivars ‘Varano’ and ‘Svevo’. By this criterion, only three bombardment experiments were successful in producing HMW-GS gene transformants, and the overall transformation frequency for these three cultivars was 0.3%. Individual transformation experiment efficiencies are shown in Table 1. No transgenic lines were obtained from cultivar ‘Latino’.

Transgene expression and inheritance

SDS-PAGE and germination in the presence of bialaphos were used to follow inheritance of the HMW-GS and *bar* transgenes, respectively. Durum wheat cultivars typically contain 2–3 HMW-GS encoded by the group 1 chromosomes of the A and B genomes. The cultivars used in our study contain only B-genome-encoded subunits. ‘Creso’ contains subunits Bx6 and By8, while ‘Svevo’ and ‘Varano’ contain subunits Bx7 and By8. Protein gel analyses of T_1 generation seed extracts identified four independently derived plants in which one or both D-genome-derived glutenin genes were also expressed, as evidenced by the presence of new proteins that co-migrate with Dx5 or Dy10 (Fig. 1). Of the four transgenic lines, DS2-127 of cv. ‘Svevo’ and DC2-10 of cv. ‘Creso’ showed the expression of all three introduced genes as manifested by bialaphos resistance of germinating embryos (Weeks et al. 1993) and the presence of both the Dx5 and Dy10 subunits in endosperm extracts. Line DV1-4 of cv. ‘Varano’ showed expression of the *bar* and *1Dx5* transgenes, but the Dy10 HMW-GS was not detected. Transgenic line DC2-65 showed co-suppression of all HMW-GS, including the endogenous ones (Fig. 1).

Co-segregation of the transgenes in progeny seed was observed in three of the four lines, indicating that both the marker and HMW-GS transgenes were integrated at a single locus. Seed protein analysis in the T_1 generation of transformant DS2-127 showed a different pattern of segregation of *1Dx5* and *1Dy10* genes. Out of seven T_1 seeds analyzed, two expressed both 1Dx5 and 1Dy10 proteins, one seed showed the expression of only the 1Dy10 subunit, and four lacked D glutenin subunits (Fig. 2). The absence of complete co-segregation suggested that there were two transgenic loci in this line: one that expressed both D-genome subunits and one that only expressed 1Dy10.

Searches for homozygotes were conducted among T_1 to T_4 generation progeny. Homozygotes for DC2-10 and DV1-4 were first detected in T_2 generation seeds while those for DC2-65 and DS2-127 were first detected in the T_3 generation. Homozygous progeny were also identified for a subline of DS2-127 showing only the expression of 1Dy10 protein. This subline was named DS2-127-Dy10.

Characterization of transgene loci

FISH analyses of metaphase chromosomes from homozygous T_4 transgenic lines showed that integration of transgenes occurred both in telomeric and centromeric regions (Fig. 3). The three plasmids were found inserted at a single locus in two lines (DC2-10, DV1-4). The third line DC2-65 had all three plasmids in each of two insertion sites in the telomeric and subtelomeric region of the same chromosome. The fourth line (DS2-127) had two transgenic loci on different chromosomes: one with the co-insertion of the three plasmids and an additional one containing only the construct with the gene encoding the 1Dy10 glutenin subunit. Segregation of these two loci allowed establishment of two sub-lines, one containing both 1Dx5 and 1Dy10 and the other containing only 1Dy10. Judging from fluorescence intensity compared to that from a single-copy of the same genes, we could hypothesize the presence of multiple copies of each plasmid.

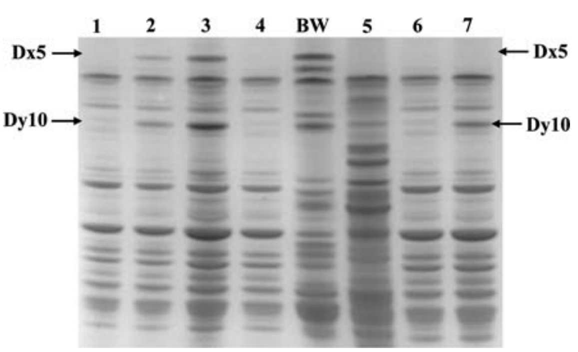

Fig. 2 SDS-PAGE of endosperm extracts showing transgene segregation in the T_1 generation of transformant DS2-127. Locations of HMW-GS Dx5 and Dy10 are indicated. Lanes 1, 4, 5, and 6 contain the HMW-GS of the non-transformed 'Svevo' parent. Lanes 2 and 3 contain, in addition to the native 'Svevo' proteins, transgene-encoded HMW-GS Dx5 and Dy10, while lane 7 contains transgene-encoded HMW-glutenin Dy10 only. Lane BW contains endosperm proteins from the non-transformed bread wheat 'Bobwhite' which include native HMW-glutenins Dx5 and Dy10

Inheritance of transgenes in the progeny derived from backcross

The chromosomal studies conducted with FISH were confirmed by the inheritance behaviour of the transgene loci in backcrossed populations. In order to study inheritance and expression of transgenes, T_4 plants from each transgenic line were backcrossed with their non transgenic parental cultivar. Protein (1Dx5, 1Dy10) segregation patterns observed in the BC1F2 progenies of DC2-10 and DV1-4 transgenic lines fitted a Mendelian expectation for a single dominant locus (3:1). The transgene-encoded HMW-GS showed co-segregation in line DC2-10. A deviation from 3:1 ratio was observed for 1Dx5 and 1Dy10 in the BC1F2 progenies of DS2-127 lines (Table 2): fewer progeny showed expression of the transgenes that expected for one or two loci.

Field trials of transgenic durum wheats

Homozygous seeds from lines DC2-10, DC2-65, DV1-4 and the subline of DS2-127 that contained only Dy10 were grown in a small field trial in one North Dakota location in the summer of 2005. Three plots of each transgenic line, their untransformed parents, 'Creso', 'Varano' and 'Svevo', and North Dakota adapted varieties 'Alzada', 'Lebsock' and 'Rugby' were planted in a randomized block design. The wheats headed between 52 and 55 days after planting and reached physiological maturity 87 to 95 days after planting. 'Alzada', 'Svevo', 'Varano' and transgenics derived from the latter two matured

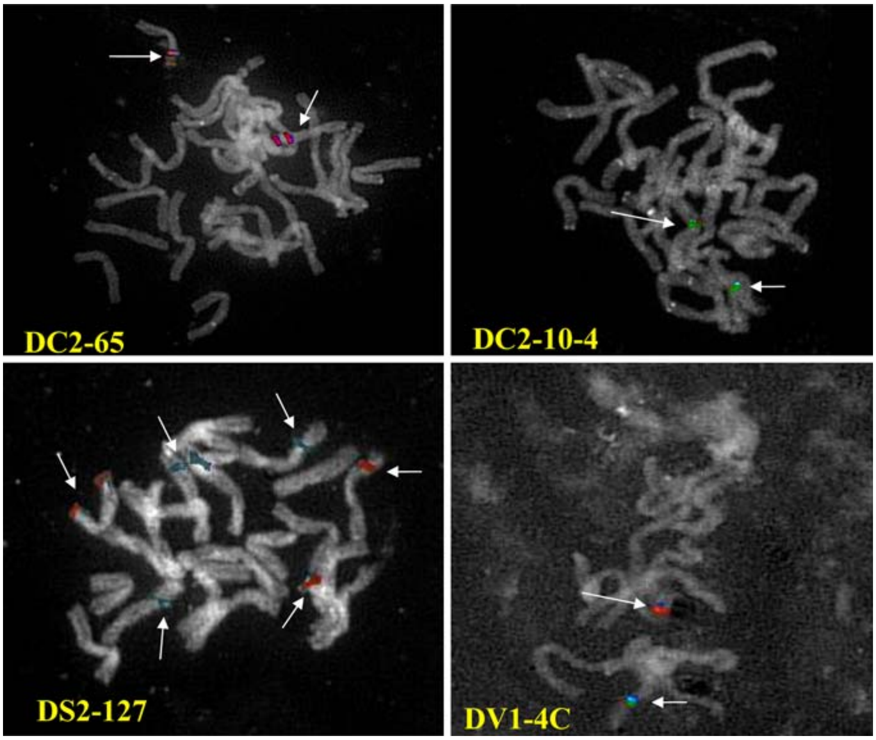

Fig. 3 FISH of four transgenic durum wheat lines produced using microprojectile bombardment. In situ hybridization was performed using as probes whole plasmids pKS-Dy10, pKS+Dx5 and Ubi:Bar directly labeled respectively with Cy5 (Blue), Cy3 (Red) and FITC (Green) by nick-translation. Fluorescence was observed by single colour. In 3 (DC2-65, Dc2-10-4, Dv1-4C) of the 4 lines all the plasmids mapped on the same chromosome, while the DS-127 line pKS+Dy10 and Ubi:Bar (respectively in green and blue) show signals also on different chromosomes. Because of the co-integration the three colours are overlapped and can not be easily identified. All the plasmids showed an amplified signal thus indicating they were present in multiple copies

Table 2 Segregation of *1Dx5* and *1Dy10* HMW-GS transgenes in BC1F2 progeny of crosses of transgenic lines with non-transgenic durum wheat parental cultivars

Transgenic lines	BC1F2 plants tested N°	HMW-GS components	Transgene segregation ratio	$\chi^2_{3:1}$
DC2-10	83	Dx5	62(+): 21(−)	0.01
		Dy10	62(+): 21(−)	0.01
DS2-127	127	Dx5	84(+): 43(−)	8.7*
		Dy10	88(+): 39(−)	9.6*
DV1-4	97	Dx5	73(+): 24(−)	0.01

+, presence of glutenin subunit; −, absence of glutenin subunit

* Significant deviation from the 3:1 ratio expected for a single dominant gene ($P < 0.01$)

earlier than ‘Creso’ and its transgenic lines. Table 3 shows the grain yield and test weight data. The North Dakota varieties performed better in terms of grain yield than the Italian varieties, as expected since the former are adapted for this environment. In most cases, the transgenic lines performed about the same as their non-transgenic parents in terms of yield. DC2-10 had higher yield and test weight than its non-transformed parent ‘Creso’. Test weights for ‘Varano’ and its transgenic derivative DV1-4 and for transgenic DC2-65 were notably lower than those of the other genotypes. The DS2-127-Dy10 line

Table 3 Field trial data averaged for replicate plots of four transgenic durum wheats and controls

Variety/line[a]	Grain yield[b] (kg/ha)	Test weight[b] (kg m^{-3})	%Protein[c] (15% M.B.)	SDS sedimentation[c] (ml)
'Alzada'	2926.7 ± 122	882.8 ± 25	n.d.	n.d.
'Lebsock'	2808.3 ± 220	934.2 ± 19	n.d.	n.d.
'Rugby'	3188 ± 352	956 ± 19	n.d.	n.d.
'Creso'	1503 ± 128	887 ± 19	12.7 ± 0.2	5.7 ± 0.5
DC2-10	**2349.7 ± 202**	**913 ± 24**	**13.1 ± 0.2**	**6.9 ± 0.4**
DC2-65	**1472.7 ± 79**	**835.8 ± 26**	**13.4 ± 0.6**	**2.0 ± 0.1**
'Svevo'	2184 ± 82	914 ± 10	13.8 ± 0.3	5.4 ± 0.5
DS2-127-DY10	**2253.3 ± 164**	**912.7 ± 20**	**15.6 ± 0.2**	**6.3 ± 0.4**
'Varano'	2268.7 ± 113	858.5 ± 14	14.1 ± 0.5	5.8 ± 0.6
DV1-4	**2215.3 ± 203**	**847.5 ± 17**	**16.2 ± 0.3**	**6.3 ± 0.8**

[a] Transgenic lines are in bold and listed immediately under their non-transgenic parent in each table

[b] Average ± standard deviations for three plots

[c] Average ± standard deviations for three plots, except for 'Svevo', for which only 2 plots were included in these analyses

M.B., moisture basis

n.d., not determined

performed, on average, the same as its non-transformed parent 'Svevo' in this trial, even though its planted seeds were from greenhouse-grown rather than field-grown plants.

Effects of the HMW subunits on flour functionality

Flours milled from 100 g of seeds from each plot of the transgenics and their parents were subjected to small scale quality tests. The protein contents of two of the transgenic flours, DV4-1 and DS2-127-Dy10, were notably higher than those of their non-transgenic parents (Table 3).

The SDS sedimentation test is used by breeders to assess the gluten strength of small flour samples (Dick and Quick 1983). Comparisons of SDS sedimentation volumes for the DC transgenic flours to that of their transgenic parent 'Creso' revealed that the presence of the D-genome subunits Dx5 and Dy10 in DC2-10 increases SDS-sedimentation volumes relative to 'Creso', while suppression of HMW-glutenin synthesis in DC2-65 markedly decreases SDS sedimentation values (Table 3). Flours from lines DV4-1 and DS2-127-Dy10 had higher SDS sedimentation volumes than those from their non-transgenic parents.

The 2-gram mixograph is used to measure differences in dough development and changes in resistance during mixing, giving information on dough strength and stability. Several parameters are measured, three of which are most important: maximum resistance achieved (peak resistance), the time taken to achieve maximum resistance (mixing time), and the rate of resistance loss after the maximum is achieved (resistance breakdown). Figure 4 shows representative traces of resistance during 30 min of mixing for each transgenic flour in comparison to that of its non-transgenic flour. The mixing times and peak resistances, indicated on each trace, are measures of dough strength, as are the widths of the traces at and after the peak resistance. The resistance breakdown is a measure of mixing tolerance. Flours from transgenics DS2-127-Dy10 and DV1-4 make slightly stronger doughs than those of their respective parents 'Svevo' and 'Varano', which have very similar mixing characteristics (Fig. 4). The effect of Dy10 is most clearly seen in increasing the width of the trace of DS2-127-Dy10 relative to 'Svevo'. The strengthening effects of Dx5 in DV1-4 are manifested as a longer mixing time and slightly higher peak resistance. Neither DS2-127-Dy10 nor DV1-4 differed from their respective parents in mixing tolerance.

The 'Creso' flour has much lower mixing tolerance than those of the other two non-transgenic Italian varieties (Fig. 4). Accumulation of Dx5 and Dy10 in its transgenic derivative DC2-10 vastly improves the mixing characteristics of 'Creso', resulting in higher

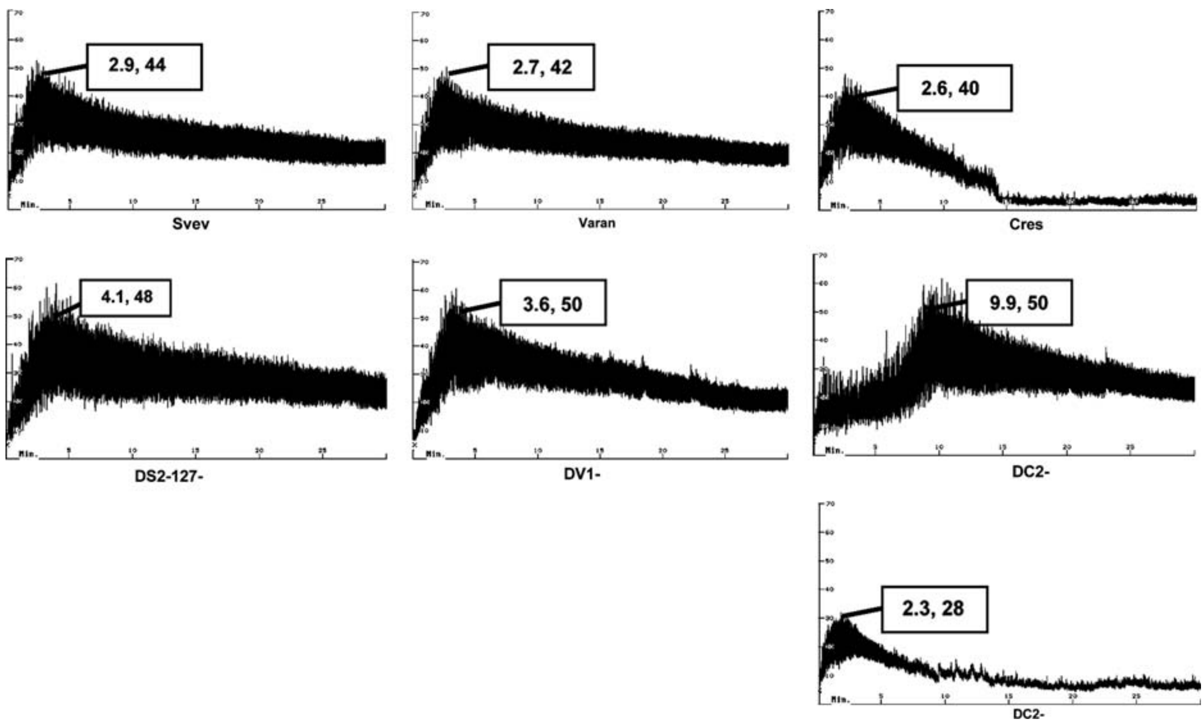

Fig. 4 Mixographs for doughs made from flours of four transgenic durum wheats and their non-transformed parents. Resistance in arbitrary units (AU) is plotted versus time for 30 min of mixing. The time to reach peak resistance (min) and height of the midline at peak resistance (AU) is shown in the box for each mixograph

peak resistance, longer mixing times, and much greater stability to resistance breakdown, as evidenced by the wider trace and decreased slope after peak (Fig. 4). In contrast, the suppression of HMW-glutenin synthesis in DC2-65 results in a much weaker dough that does not develop further after hydration.

Discussion

In the last decade, transformation experiments with HMW-GS in wheat have been published by several research groups (reviewed and referenced in Jones 2005; Blechl et al. 2007). In order to study their effects on dough properties, HMW-GS genes have also been used to transform tritordeum (Rooke et al. 1999a), maize (Sangtong et al. 2002), and rye (Alpeter et al. 2004). The HMW-GS genes used were *1Dx5*, *1Ax1* or a hybrid between the *1Dy10* and *1Dx5* genes (Blechl and Anderson 1996). Stable integration expression of the *1Dy10* gene has only been recently reported by Altpeter et al. (2004) in rye and by Blechl et al. (2007) in bread wheat. In our study, transformation of four commercial durum wheat cultivars with HMW-GS genes *1Dx5* and *1Dy10* was carried out in order to study the expression of the two genes in different genotypes. Four transgenic lines were obtained. Two transgenic events expressed the 1Dx5 and 1Dy10 subunits; one transgenic event expressed the 1Dx5 subunit, and one transgenic event showed a co-suppression of transgenes *1Dx5* and *1Dy10* and the native genes encoding the endogenous HWM glutenin subunits.

Transformation efficiencies varied widely among the individual experiments and ranged from 0.1% and 1%, with an average of 0.3% (excluding the results for 'Latino'). These efficiencies are similar to those obtained in previous studies in durum wheat using the *bar* gene as the marker gene and bialaphos as the selection agent (Bommineni et al. 1997; He et al. 1999; Gadaleta et al. 2006) or using *pmi* as the marker gene and mannose as the selection agent (Gadaleta et al. 2006). Tosi et al. (2004) used phosphinothricin as a selective agent for the presence of the *bar* gene and obtained transformation efficiencies of 0.6–3.1% in cultivars 'Svevo' and 'Ofanto'.

In this study, we did not obtain any transformants in cultivar 'Latino', most likely because only 20% of the calli regenerated into plants. In contrast, cultivars 'Svevo' and 'Creso' had regeneration percentages of 97% and 96% respectively (Gadaleta et al. 2002). These data demonstrate that durum wheat genotype is an important factor for plant regeneration and transformation success.

Transmission and expression of the transgenes were investigated in the T_2, T_3 and T_4 generations in each of the four transgenic lines and in backcrosses to non-transgenic parents. Homozygous progeny were derived by selfing of each line. Expression of the glutenin transgenes was stable through the T_4 generation of selfing. The stability of transgene expression was also demonstrated by Shewry et al. (2006) in a set of transgenic wheat lines expressing HMW-GS transgenes in comparative field performance over three years, confirming that transgenic lines are not necessarily less stable than those produced by conventional breeding.

Examination of transgene expression in selfed progeny of backcrosses of DC2-10 and DV1-4 revealed that ¾ contained both glutenin transgenes, as expected for co-segregation of a single locus. However, we also found that fewer than expected selfed progeny from the back-cross of DS2-127 to its parent 'Svevo' had detectable levels of 1Dx5 and 1Dy10 in their seeds. This may be due to poor transgene transmission or silencing in some of the progeny. Inheritance of transgenes can be erratic. Srivastava et al. (1996) observed the loss of transgenes in T_3 wheat plants following loss of expression in prior generations. Sangtong et al. (2002) found that the wheat *1Dx5* gene was poorly transmitted through the pollen of transgenic maize plants. Poor transmission of integrated transgenes in the progeny of some transgenic lines has been observed also in maize (Spencer et al. 1992; Register et al. 1994) and barley (Cho et al. 1999). These results emphasize the need for confirming transgene inheritance and expression stability before the products of biotechnology can be integrated into breeding strategies.

Although transformation of crop genomes by biolistic methods has become routine, the mechanisms of transgene integration into the host genome are still not understood. Possible mechanisms of transgene integration include recombination via non-homologous end joining during DNA double strand break repair (Kohli et al. 1998). Some clues may be obtained by studying the structures of transgene loci that result from integration. Traditionally such structures are characterized using Southern analysis. Recent results obtained in various crops indicate that FISH is also a powerful tool for the detection and characterization of transgene locus structures. In most reports, the majority of transformants exhibit transgene integration into distal chromosome regions (Wang et al. 1995; Pedersen et al. 1997; Salvo-Garrido et al. 2001). We have applied FISH analyses to our set of four transgenic durum wheats and found that the sites of transgene integration were randomly distributed throughout the genome and occurred both in telomeric regions (lines DC2-65, DS2-127) and centromeric regions (lines DC2-10 and DV1-4). These results are in accord with those of Abranches et al. (2000); their analysis on wheat transgenic lines also showed a lack of preference for integration of transgenes at distal chromosome sites. Barro et al. (2003b) used FISH to show that the transgene loci in two lines of transformed tritordeum (a durum wheat/barley hybrid) co-localized with *Hordeum chilense* DNA translocations into the middle of wheat chromosome arms. They employed *Hordeum*-specific satellite DNAs as probes to visualize the translocations. Jackson et al. (2001) did not detect such rearrangements in their transgenic wheat plants. The probes we used in this study were not designed to detect such rearrangements.

Traditionally transgene locus number and their inheritance are scored by segregation analysis of transgenic phenotype and/or genotype (Svitashev and Somers 2001). However, because of transgene silencing and tissue-culture-mediated chromosomal rearrangements, the determination of transgene locus number is often difficult (Svitashev et al. 2000). The determination of transgenic locus number through FISH overcomes the problems linked to phenotypic and genotypic segregation analysis and also allows easy identification of homozygotes (Barro et al. 2003b). In our transgenic lines, the number of integration sites ranged from 1 to 2 per homologous chromosome. The presence of more than one transgene locus is frequently observed in plants transformed via microprojectile bombardment (Blechl and Anderson 1996; Pedersen et al. 1997, Jackson et al. 2001; Rooke et al. 2003). The three plasmids were found to be co-integrated into the same genomic transgene locus in all the four transgenic lines, a co-transformation frequency of 100%. Co-transformation and co-expression frequencies of selectable marker and HMW-GS transgenes in durum wheat were reported at 67% by He et al. (1999). The high frequency of co-integration of biolistically introduced DNAs carried on separate plasmids may be related to ligation of introduced DNA and concatamer formation (Pawlowski and Somers 1996; Kohli et al. 1998).

Genetic engineering of plants sometimes results in transgene silencing after integration into the host genome, which may be related to a defence mechanism against foreign DNA expression (Kumpatla et al. 1998). In our work, two out of four transgenic events showed silencing for one or all the HMW-GS. While FISH analysis showed that line DV1-4 was co-transformed with the three different plasmids containing *1Dx5*, *1Dy10* and *bar* genes, only the *1Dx5* and *bar* genes produced protein products. This indicates that either the *1Dy10* gene was silenced or that it was altered in some way that prevented translation. For line DC2-65, FISH results indicated co-transformation with three genes, but only the *bar* gene was expressed. Expression of both HMW-GS transgenes and endogenous HMW-GS genes was suppressed, and the co-suppression was found to be genetically stable in the generations analysed from T_1 to T_4. The phenomenon of transgene silencing has often been reported for HMW-GS transgenes in hexaploid wheat (Altpeter et al. 1996; Blechl et al. 1998; Alvarez et al. 2000).

The transgenic wheats produced in this study were useful in studying the separate and combined effects of subunits 1Dx5 and 1Dy10 in genetic backgrounds that lack the D-genome encoded HMW-GS. SDS sedimentation tests showed that flours from all the transgenics except the line that exhibited co-suppression had higher gluten contents than those of their non-transformed parents. SDS sedimentation volumes are influenced by both the quantity and quality of gluten proteins (Carter et al. 1999), so part of the increase in lines DV4-1 and DS2-127-Dy10, could be due to their higher overall protein contents. Protein content is a quantitative character strongly influenced by environmental conditions (Blanco et al. 2006), so data from different locations and years would be needed to discern whether the higher values obtained for the DV4-1 and DS2-127-Dy10 are due to genotype, environment or their interaction. Increases in SDS sedimentation volumes have been observed in some transgenic flours expressing *1Ax1* transgenes (Barro et al. 2003a) and in some expressing *1Dx5* and/or *1Dy10* transgenes (Blechl et al. 2007). However, high levels of Dx5 and/or Dy10 typically result in reductions in SDS-sedimentation volumes (Barro et al. 2003a; Darlington et al. 2003; Rakszegi et al. 2005; Blechl et al. 2007). The increases in SDS sedimentation volumes we observe in our transgenic durum lines are probably due to the moderate levels of expression of the *1Dx5* and *1Dy10* transgenes in genetic backgrounds whose seeds only contain two other HMW-GS. Thus, their HMW-GS compositions are most similar to those of the B102 and B72-8 lines analyzed by Popineau et al. (2001), Barro et al. (2003a) and Darlington et al. (2003). Co-suppression of HMW-GS, such as in our line DC2-65, results in decreased SDS sedimentation volumes, as was also observed by Alvarez et al. (2001).

The effects of Dx5 and/or Dy10 on mixing were seen in changes in mixograph traces for flours from transgenics DV1-4, DS2-127-Dy10 and DC2-10 compared to those of their non-transformed parents. The effects are most apparent for DC2-10, which contains both D-genome encoded subunits. The parent 'Creso' has poor mixing tolerance, while DC2-10 has good mixing stability and much increased strength, as evidenced by the high values for mixing time and peak resistance. Such improvements are also evident in some hexaploid wheat transgenics that have moderate levels of over-expression of Dx5 and Dy10 (Blechl et al. 2007). The effects of the individual subunits on the mixing behaviour of 'Varano' and 'Svevo' were more subtle. The main effect of Dy10 is to increase bandwidth, a result also seen when Dy10 levels were moderately increased in transgenic hexaploid wheat (Blechl et al. 2007) and when Dy10 was incorporated by reduction/oxidation into a transgenic flour with 2.4x normal levels of Dx5 (Butow et al. 2003). The effect of Dx5 in 'Varano' was a modest increase in mixing time and peak resistance. This result is similar to the effect of Dx5 in durum wheat 'Ofanto' (He et al. 1999), but was not found when it accumulated to higher levels in durum cultivar 'L35' or when Dx5 was over-expressed in wheats that contained 5 HMW-GS, including Dx5 and Dy10. In those backgrounds, increases in Dx5 led to doughs that were difficult or impossible to mix in the 2-gram mixograph without blending with weaker flours (Barro et al. 1997; Rooke et al. 1999b; He et al. 1999; Alvarez et al. 2001; Popineau et al. 2001; Blechl et al. 2007). Our results and those cited here show using transformation to add Dx5 and/or Dy10 in moderate amounts, roughly equivalent to those of native HMW-GS, to durum wheat improves dough mixing properties, which could lead to improved quality for making breads or pasta.

Knowledge of the behaviour of useful transgenes, along with the possibility of expressing them in the desired varieties, will play key roles in the success of transgenic technology in plant breeding. The results reported here demonstrated that transformation of different durum wheat genotypes and stable integration and expression of important genes such as those that encode HMW-GS can be obtained.

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